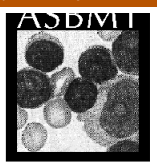

Comparison of the distribution of progenitor cells in G-CSF–mobilized peripheral blood and steady-state bone marrow after counterflow centrifugal elutriation

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ABSTRACT

Blood-derived progenitor cells obtained following mobilization with granulocyte colony-stimulating factor (MoPBSC) are increasingly being used as an alternative to bone marrow (BM) in allogeneic stem cell transplantation. The higher numbers of mature T lymphocytes in MoPBSC grafts may increase the risk of (chronic) graft-vs.-host disease. Counterflow centrifugal elutriation (CCE) is an effective method for T-cell depletion of BM grafts. The elutriation characteristics of steady-state BM and MoPBSC were compared using a CCE procedure in which fractions were obtained after small incremental increases in flow rate with constant centrifugal force. Counterflow centrifugal elutriation experiments with MoPBSC from six healthy volunteers showed that 54% of all cells collected were recovered in the ≤15 mL/minute fractions, whereas experiments with mononuclear BM cells from five healthy volunteers resulted in recovery of 52% of collected cells from the ≥19 mL/minute fractions. The peak concentrations of $CD34^+$ cells were found in the same fraction (18 mL/minute), but more $CD34^+$ cells from MoPBSC were recovered from the small (≤16 mL/minute) fractions (54% for MoPBSC, 26% for BM; $p = 0.08$). The small $CD34^+$ cells from BM were more frequently lacking CD38 and human leucocyte antigen-DR expression than the small $CD34^+$ cells from MoPBSC. Mature T-cells ($CD3^+$) in BM and MoPBSC samples had similar CCE features, as did early (long-term culture initiating cells, high-proliferative potential colony-forming cells) and more mature (colony-forming units granulocyte/macrophage, BFU-e) hematopoietic progenitor cells. The results of this study suggest that T-cell depletion by CCE of MoPBSC as compared to BM products, may lead to a greater loss of $CD34^+$ cells, but not of immature hematopoietic progenitor cells.

KEY WORDS

Elutriation • CD34 • Bone marrow • Peripheral blood • Stem cells

INTRODUCTION

Blood-derived progenitor cells are being used increasingly as an alternative to bone marrow (BM) as a source of stem cells for human auto- and allografting [1–3]. The use of hematopoietic growth factors is essential to increase the number of circulating progenitors in the peripheral blood in order to allow rapid and efficient collection of stem cells from normal donors [1–4]. For this purpose, Filgrastim, a granulocyte colony-stimulating factor (G-CSF), has been used in most of the published allograft studies as the growth factor of choice [3–6]. The use of such G-CSF mobilized progenitor cells (MoPBSC) for infusion into myelo-ablated recipients has resulted in consistent and rapid engraftment.

The number of T cells in a typical G-CSF mobilized peripheral blood graft (MoPBSC), however, is up to 10-fold higher than in an average BM graft [1,3–8]. This observation raises the possibility that MoPBSC would be associated with a higher risk of graft-vs.-host disease (GVHD) than bone-marrow allografts [8,9]. Clinical data do suggest an increased incidence for the chronic variant of GVHD [3,7–10]. Depletion of T cells from bone marrow grafts has been successful in decreasing the incidence and severity of GVHD, both in matched and mismatched allografts [11–13].

Counterflow centrifugal elutriation (CCE) separates cells according to size and, to a much lesser degree, density [14]. This technique has been used very successfully to remove T lymphocytes in clinical BM allografting [15–18]. CCE-processed BM cells have been reported to be enriched for assayable hematopoietic progenitor cells (HPC), but it has also been reported that the recovery of very early HPC (e.g.,

long-term culture initiating cells [LTC-IC]) may be poor compared to more mature HPC (e.g., colony-forming units granulocyte/macrophage [CFU-GM]) [19–21]. We postulated that the use of MoPBSC depleted of T cells via CCE might be an attractive method to decrease the risk of severe GVHD after transplantation. In this study, the elutriation profiles of MoPBSC are compared with those of steady-state (nonmobilized) BM.

MATERIALS AND METHODS

Donors and collections

Donors for this study were healthy paid volunteers who donated either BM or mobilized peripheral blood components. All had given informed consent according to guidelines established by the Institutional Review Board of the Methodist Hospital of Indiana. Bone marrow samples of 50–70 mL were aspirated from the posterior iliac crest and collected into heparin. Peripheral blood cells were collected by leukapheresis with a COBE Spectra on day 5, following four daily s.c. injections of G-CSF (Neupogen, Thousand Oaks, CA) 10 μg/kg. MoPBSC were collected into acid-citrate-dextrose-A. Typical collections processed a volume equal to two times blood volume, or a total of 10L.

Leukocyte separation and red-cell depletion for MoPBSC

The collected MoPBSC were diluted with calcium- and magnesium-free Hanks' balanced salt solution (HBSS) (Whittaker, Walkersville, MD) and centrifuged at 400 *g* for 5 minutes. The supernate was then removed and replaced by red-cell lysing solution (0.1% $KHCO_3$, 0.85% NH_4Cl) for 5 minutes. The cells were washed twice in HBSS and resuspended in 20–40 mL of HBSS with 2% fetal bovine serum (FBS) (Hyclone, Logan, UT). Samples of this suspension were then diluted in Isoton II Diluent (Coulter Diagnostics, Hialeah, FL) and counted with a Coulter CBC5 cell counter.

Mononuclear cell separation for BM

BM samples were diluted five times with Ca- and Mg-free HBSS. Mononuclear cells were isolated by density gradient centrifugation (340 *g* for 20 minutes) over Ficoll-Hypaque (1.077 g/dl; Pharmacia, Piscataway, NJ). Cells were then washed once with HBSS and resuspended in 20–40 mL HBSS with 2% FBS. Samples were taken for cell counts.

Counterflow centrifugal elutriation procedure

Cell suspensions were loaded into a Beckman JE-5.0 elutriation rotor with a 4-mL standard chamber (Beckman Instruments, Palo Alto, CA) after sterilization by autoclaving (121°C for 20 minutes). Loading was performed at 18°C with a flow rate of 5 mL/minute and a rotor speed of 2600 rpm (relative centrifugal force 651–946 *g*). The elutriation medium was 0.9% Sodium Chloride Injection USP (Baxter, Deerfield, IL). A Masterflex peristaltic pump (Cole-Parmer Instrument, Barrington, IL) was used to maintain a constant and precise flow rate. After the cells had been loaded into the chamber, the rotor speed was decreased to 2300 rpm (510–741 *g*). Subsequently, the flow rate was slowly increased to 14 mL/minute with rotor speed held constant. A volume of 50 mL was eluted. Then stepwise, the flow rate was increased incrementally by 1 mL/minute, and volumes of 50 mL were collected at 15, 16, 17, 18, 19, 20, 21, 22, and 23 mL/minute. Finally, the rotor was turned off and the remaining cells were eluted (R/O fraction). The cells collected in each fraction were centrifuged and resuspended in phosphate-buffered saline (PBS) with 2% FBS and 1 mM ethylene diamine tetraacetic acid (EDTA).

Methylcellulose assays

Cells fractionated by CCE, or cells obtained from long-term cultures were plated in 35-mm petri dishes (Corning, Cambridge, MA) in a total volume of 1 mL standard methylcellulose complete medium containing 0.9% methylcellulose, 30% FBS, 1% bovine serum albumin, 100 μmol/L 2-mercaptoethanol, 3 U/mL erythropoietin, 2 mM L-glutamine, and 5% phyto-hemagglutinin treated leukocyte conditioned medium (StemCell Technologies, Vancouver, British Columbia, Canada). All cultures were incubated at 37°C in fully humidified air with 5% CO_2. At 14 days, cultures were started in duplicate and scored with an inverted microscope for the presence of erythroid bursts (BFU-e), GM colonies (CFU-GM), and mixed-cell colonies (CFU-GEMM). BFU-e (containing >200 cells) were identified on the basis of their orange-red color. Colonies of >50 nonerythroid cells were scored as GM colonies. Colonies containing >200 cells composed of both erythroid and nonerythroid elements were scored as mixed-cell colonies. High-proliferative potential colony-forming cells (HPP-CFC) were scored after 28 days in culture, and were distinguishable as large (>0.5 mm and frequently up to 3 mm in diameter), dense, late-appearing colonies which were composed primarily of granulocytes and a smaller number of monocytes.

Long-term culture initiating cells

As described, Stroma-noncontact cultures were used to assay for long-term culture initiating cells (LTC-IC) [20]. Stromal layers, which consisted of 1×10^5 cells and were derived from the R/O fraction of the same donor, were put in the upper well of 24-well transwell plates (0.4 μ-pore polycarbonate membrane; Costar, Cambridge, MA). 1×10^5 fractionated cells were plated in the bottom wells of the transwell plates. No cytokines were added to the long-term culture complete media (StemCell Technologies), which consisted of alpha-minimum essential medium with 12.5% FBS, 12.5% horse serum, 10^{-4}M 2-mercaptoethanol, 2mM L-glutamine, 0.2mM i-inositol, 20μM folic acid, and 10^{-6}M water soluble hydrocortisone. The cultures were maintained in fully humidified air at 37°C and 5% CO_2 for 5 weeks. At weekly intervals the cultures were fed by removing half of the cell-free supernatant from the top or bottom wells and replacing with fresh complete media. Nonadherent cells were recovered from the bottom wells after 5 weeks and were assayed in the CFU-GM assay for the presence of LTC-IC.

Flow cytometry studies

The FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA) equipped with an argon laser with emission at 488 nm was used. Data acquisition was accomplished with the Lysys 2.0 software package after gating of cell populations defined by their light-scatter characteristics. A minimum of 20,000 events were analyzed for each sample. For surface antigen detection, cells were washed once with

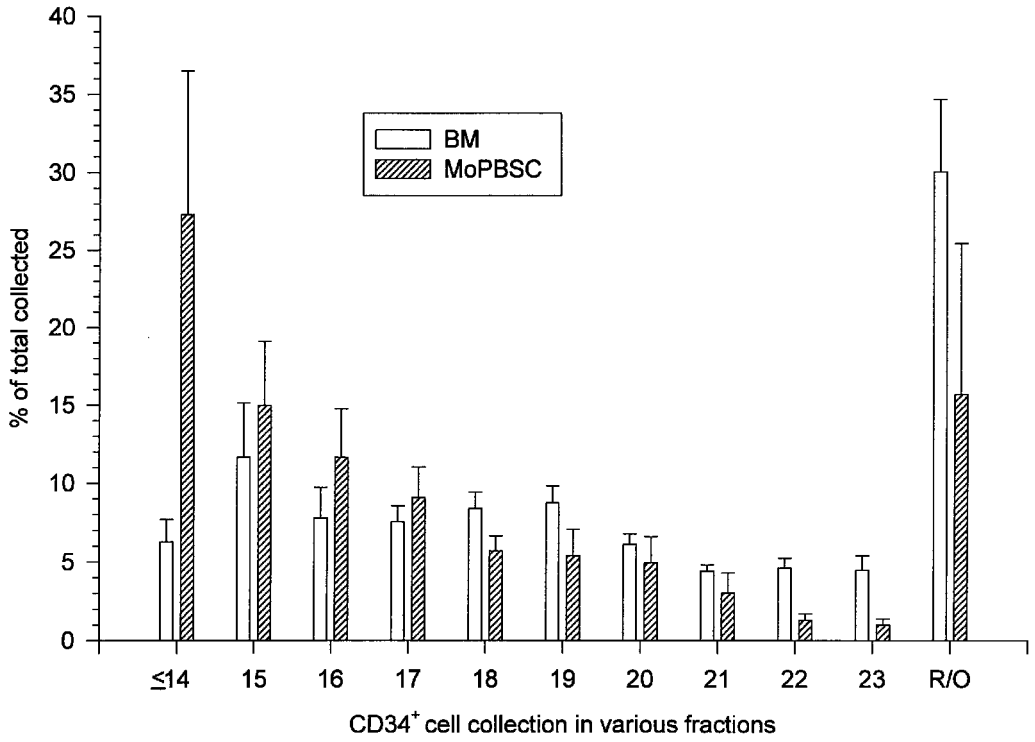

Figure 1. CD34 + cells collected from each elutriation fraction

Results are expressed as percentages of total collected CD34+ cells. Bars indicate SE.

PBS (containing 2% FBS and 1mM EDTA), and resuspended to 5–10×10^6 cells/mL in PBS. 100 μL cells were pelleted and 10 μL of the appropriate antibody was added. After incubation for 30 minutes in the dark at 4°C, the cells were washed twice with PBS, fixed with 1% paraformaldehyde, and analyzed for fluorescence. The antibodies used were: CD34-PE, CD3-PE, CD11b-PE, CD15-FITC, IgG_1-PE, and IgG_1-FITC (Becton Dickinson), CD38-FITC, HLA-DR-TC, and IgG_1-TC (Caltag, South San Francisco, CA).

Statistical anal ysis

The statistical significance of data obtained was tested with the two-tailed *t*-test. *p*-values of <0.05 were considered significant.

RESULTS

Nonfractionated samples and cell loss during CCE

Five volunteers, between the ages of 21 and 30 years, donated bone marrow for this study. Six different volunteers, between the ages of 20 and 32 years, donated G-CSF mobilized peripheral blood. The mononuclear BM cells contained 65.4 ± 5.2% lymphocytes and 28.3 ± 3.4% myeloid/monocytic cells; the MoPBSC products contained 66.2 ± 4.3% lymphocytes and 33.8 ± 3.8% myeloid/monocytic cells. These 11 specimens were subjected to the CCE procedure as described in Methods. The recovery rates for BM and MoPBSC cells were not significantly different ($p = 0.68$). About one third of the loaded cells were lost during the CCE procedure with recoveries ranging from 37 to 85%. Because of this cell loss, all further results were expressed as percentages of total cells collected.

Elutriation pr ofiles

BM and MoPBSC were subjected to CCE with a constant centrifugal force (2300 rpm) and an increasing counterflow rate with incremental steps of 1 mL/minute. Fractions were obtained at each mL/minute interval between ≤14 and 23 mL/minute; subsequently an R/O fraction was obtained. In the BM group, 17 ± 3% (mean ± SE) of all cells were collected in the ≤14 mL/minute fraction, a similar percentage in the 15 mL/minute fraction, and 34 ± 7% in the R/O fraction. Each of the other eight fractions contained only 3.1 to 5.8% of the total cells collected. In contrast, more cells were collected in the smaller cell fractions from the MoPBSC group: 49 ± 9% of the cells were obtained from the ≤15 mL/minute fractions, and fewer in the R/O fraction (21 ± 11%). Again, fractions 16–23 each contained only between 2.2 and 7.1% of all cells collected.

Distribution of CD34 + cells

BM cells loaded into the CCE centrifuge contained 3.3 ± 0.5% (mean ± SE) $CD34^+$ cells, whereas MoPBSC contained only 1.5 ± 0.5% $CD34^+$ cells. After elutriation, the BM cells were enriched for $CD34^+$ cells in fractions 16–21, and depleted in fractions ≤14 and 15. The peak concentration of $CD34^+$ cells was found in fraction 18 mL/minute. With MoPBSC, enrichment for $CD34^+$ cells occurred in fractions 17–20; the peak in fraction was again found in fraction 18 mL/minute. When *concentrations* of $CD34^+$ cells were compared, no clear differences in elutriation pattern were observed between BM and MoPBSC. When the *total numbers* of $CD34^+$ cells per collected fraction were considered, however, $CD34^+$ cells from MoPBSC were elutriated, on average, at a lower counterflow rate than $CD34^+$ cells from BM (Fig. 1).

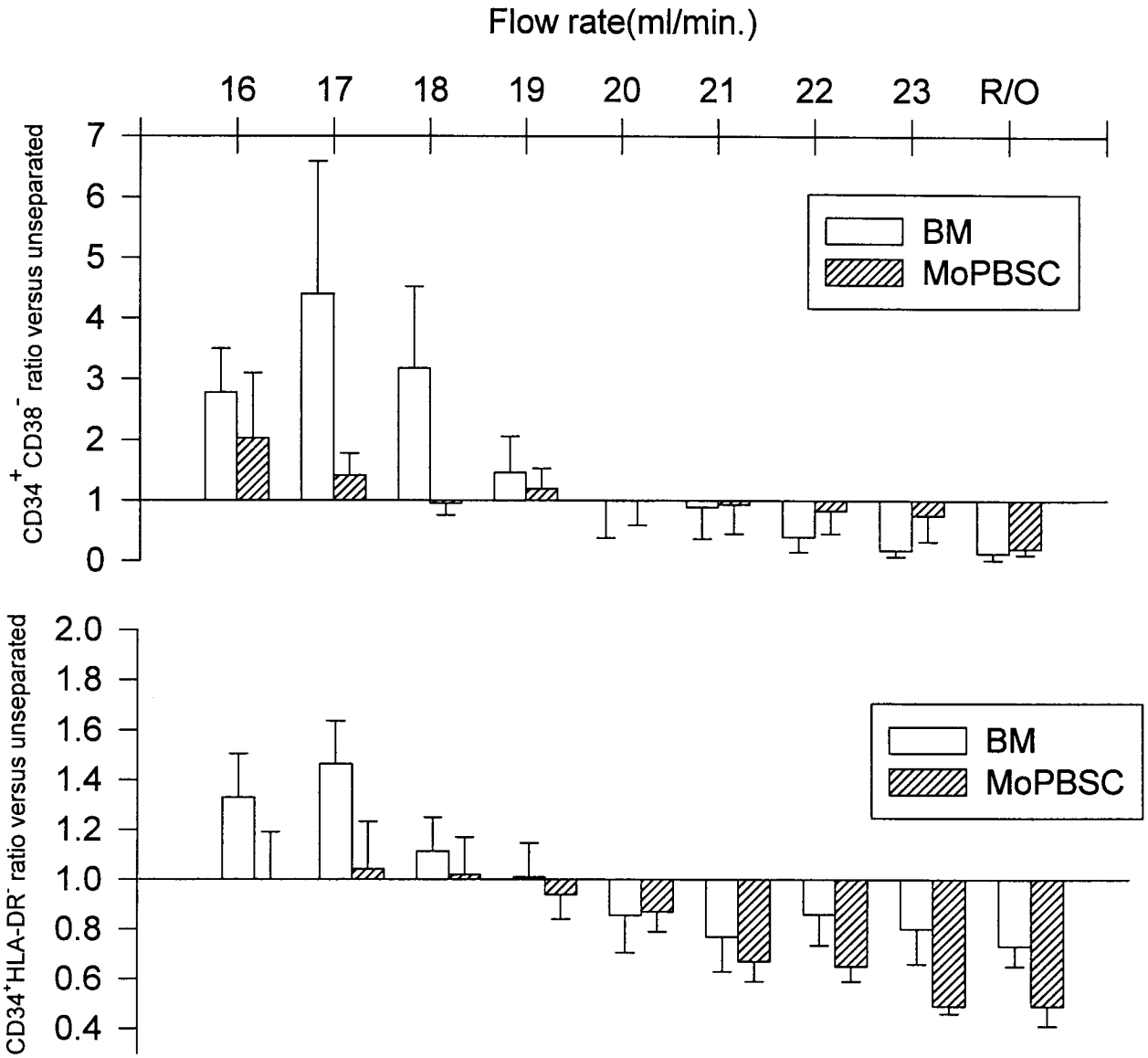

Figure 2. Populations of CD34 $^{+}$CD38- and CD34 $^{+}$DR- cells in each elutriation fraction

Results are expressed as the ratios of $CD34^{+}CD38^{-}$ vs $CD34^{+}CD38^{+}$ and $CD34^{+}DR^{-}$ vs. $CD34^{+}DR^{+}$ cells. In each case the ratio of the cells loaded was arbitrarily set to be 1. Bars indicate SE.

The number of $CD34^{+}$ cells from fractions ≤16 mL/minute, as a percentage of all $CD34^{+}$ cells collected, was greater for MoPBSC than for BM (54% vs. 26%), although the difference did not reach significance ($p = 0.08$).

Unseparated BM and MoPBSC samples were double-stained for CD34 and CD38, and for CD34 and HLA-DR. Among the $CD34^{+}$ cells from BM, 1.24 ± 0.38% were $CD38^{-}$ (mean ± SE) and 11.9 ± 2.3% were HLA-DR^{-}; among unseparated MoPBSC $CD34^{+}$ cells, 5.6 ± 2.4% were $CD38^{-}$, and 11.2 ± 1.6 % were HLA-DR^{-}. Following elutriation, BM and MoPBSC showed similar patterns, with enrichment of $CD34^{+}/CD38^{-}$ and $CD34^{+}/DR^{-}$ cells in the low flow-rate fractions (≤18 mL/minute), and progressive depletion of such cells from the larger cell fractions (Fig. 2). Among the smaller cells (fractions ≤18 mL/minute), BM had a significantly higher proportion of $CD34^{+}CD38^{-}$ cells than MoPBSC ($p < 0.03$). The same was true for $CD34^{+}DR^{-}$ cells ($p = 0.05$).

Distribution of CD3 $^{+}$ cells

The CD3 antigen was used as a marker for mature T lymphocytes in the various stem cell products. Mononuclear BM contained 20.0 ± 2.6% and MoPBSC contained 43.3 ± 6.3% $CD3^{+}$ cells. After elutriation, BM cells showed enrichment (in concentration) of $CD3^{+}$ cells in the ≤14, 15 mL/minute (peak) and 16 mL/minute fractions, and progressive depletion in the larger-cell fractions. MoPBSC showed a similar pattern of enrichment in fractions ≤14,15 mL/minute (peak) and 16 mL/minute, and progressive depletion beyond the 18 mL/minute fraction. When *total numbers* of collected

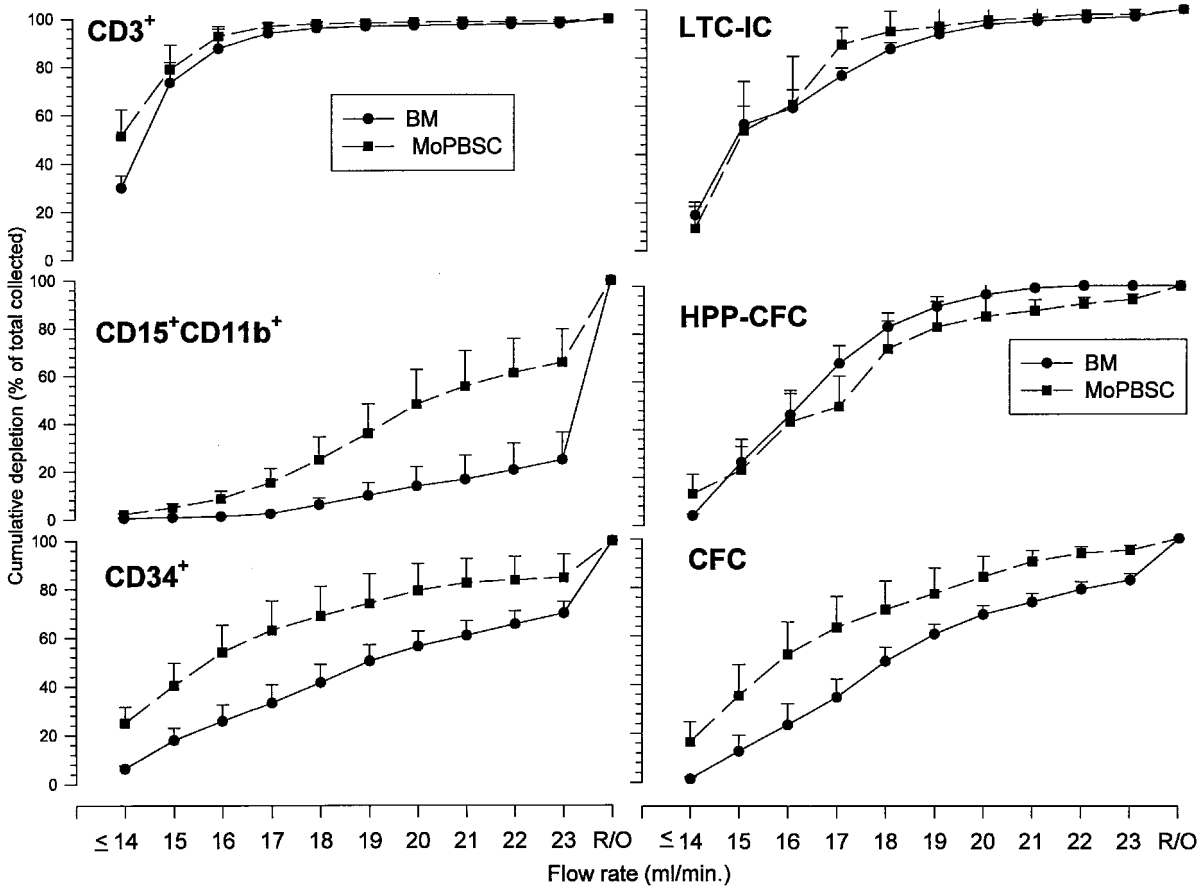

Figure 3. Cumulative depletion of various cell populations during the elutriation process
Bars indicate SE.

$CD3^+$ cells were considered, the cumulative depletion curves for BM and MoPBSC were virtually superimposable (Fig. 3).

Matur e myeloid cells

Fluorescence for the combination $CD15^+CD11b^+$ was used as a marker for mature myeloid cells. The BM mononuclear cells loaded into the elutriation chamber contained 34.4 ± 4.7%, whereas the MoPBSC specimens contained 26.8 ± 5.9% $CD15^+CD11b^+$ cells. After elutriation, progressive enrichment occurred in BM fractions ≥18 mL/minute and MoPBSC fractions ≥17 mL/minute. The cumulative depletion of $CD15^+CD11b^+$ cells proceeded more rapidly with MoPBSC than with BM (Fig. 3).

Hematopoietic progenitor cells

For this study we added the numbers of BFU-e, CFU-GM, and CFU-GEMM to arrive at the number of "more mature" myeloid progenitor cells (colony-forming cells [CFC]). HPP-CFC and LTC-IC were used to quantify early progenitor cells. The concentration of CFC in the BM and MoPBSC specimens loaded into the elutriation chamber was 488 ± 89 and 246 ± 64 (mean ± SE per 10^5 cells), respectively. After elutriation, both the BM and MoPBSC specimens showed increased CFC *concentrations* in the 16–20 mL/minute fractions. When the *total numbers* of CFC collected were studied, MoPBSC samples appeared to have a larger proportion of CFC in the smaller-cell fractions (≤ 16 mL/minute) than BM samples (Table 1; Fig. 3). The difference, however, was not significant ($p = 0.21$).

HPP-CFC were more frequent in the BM specimens (16.3 ± 5.5 per 10^5 cells) than in the MoPBSC samples (5.1 ± 2.6 per 10^5 cells). The elutriation profiles for BM and MoPBSC were very similar with enrichment in the 16–18 mL/minute fractions for BM, and the 17–20 mL/minute fractions for MoPBSC. Cumulative depletion profiles for BM and MoPBSC were superimposable (Fig. 3).

LTC-IC were clearly more frequent in the BM samples (43.3 ± 35.6 per 10^5 cells) than in MoPBSC samples (1.8 ± 1.3 per 10^5 cells). Enrichment in *concentration* was found for BM in the 15–19 mL/minute fractions, and for MoPBSC in the 15–18 mL/minute fractions. Cumulative depletion profiles for BM and MoPBSC were, again, superimposable (Fig. 3).

DISCUSSION

This study confirms results previously obtained by several groups demonstrating that CCE is capable of subdividing $CD34^+$ cells from BM on the basis of the $CD34^+$ cell maturation

Table 1. *Number of CFC in the various elutriation fractions as percentage of total CFC collected*

Fraction	BM	MoPBSC
<14 mL/minute	1.3 ± 0.7*	16.8 ± 8.1
15	11.3 ± 5.3	18.4 ± 6.0
16	10.8 ± 2.1	16.8 ± 5.0
17	11.3 ± 1.2	10.9 ± 1.8
18	14.8 ± 2.3	7.4 ± 1.8
19	11.2 ± 2.1	6.6 ± 2.6
20	8.1 ± 1.0	7.1 ± 3.0
21	5.1 ± 1.0	6.4 ± 4.2
22	5.3 ± 1.0	3.4 ± 1.8
23	3.8 ± 0.6	1.3 ± 0.6
R/O	17.2 ± 2.5	5.0 ± 2.2

**Mean ± standard error (SE).*

level. Small $CD34^+$ cells appear more likely to be CD38 negative or HLA-DR negative, or both [20,22,23], and more capable of forming early hematopoietic colonies such as HPP-CFC and LTC-IC [22–26]. The current study found a pattern quite similar to BM for G-CSF mobilized PBSC from healthy volunteers. The smallest $CD34^+$ cells from MoPBSC appeared to be the most immature. Additionally, $CD34^+$ cells from MoPBSC and those from steady-state BM appeared to peak in the same flow-rate fraction (fraction 18 mL/minute, Fig. 1). The distributions of $CD34^+$ $CD38^-$ and $CD34^+DR^-$ subpopulations were also quite similar (Fig. 2).

Furthermore, the various elutriation fractions of BM and MoPBSC contained proportionate numbers of CFC. More specifically, the number of HPP-CFC was somewhat higher in BM than MoPBSC, but the distribution over the various elutriation fractions was similar (Fig. 3). The LTC-IC concentration was markedly higher in BM than in MoPBSC; however, the distributions over the various elutriation fractions were again similar (Fig. 3). These LTC-IC results are in partial disagreement with studies by other groups [27–29]. Pettengell *et al*. found more LTC-IC in leukapheresis products obtained after mobilization with chemotherapy and G-CSF than in steady-state BM [27]. Prosper *et al*. noted a 59-fold increase in the concentration of LTC-IC in leukapheresis products mobilized with G-CSF [28]. Two possible explanations can be offered for this apparent discrepancy. Major differences exist in the methodology for LTC-IC detection. In the studies cited, identical feeder layers were used for BM and MoPBSC [27–29]. In the current study, cells from the R/O fractions were used as feeder cells. The R/O fraction cells from MoPBSC may support the proliferation of LTC-IC more poorly than those from BM. Also, there may be differences in the LTC-IC themselves. Prosper *et al*. showed that LTC-IC from MoPBSC mobilized with G-CSF, many had a limited proliferation potential [28,29]. These LTC-IC were often found in the $CD34^+/DR^+$ cell fraction, and were capable of maintaining hematopoiesis for 5, but not 8, weeks [28]. A much smaller proportion of LTC-IC are found in the $CD34^+/DR^-$ cell fraction. These LTC-IC are able to maintain hematopoiesis for 8 weeks, and are more comparable to LTC-IC from steady-state BM [29].

Our study shows that $CD34^+$ cells from steady-state BM and G-CSF–mobilized PBSC from healthy volunteers have similar elutriation characteristics. One notable difference is that $CD34^+$ cells from MoPBSC appear to have a somewhat smaller average size than $CD34^+$ cells from BM (Fig. 1). These findings are in contrast with findings obtained by our group when studying PBSC from breast cancer patients following mobilization with the combination of cyclophosphamide, VP-16, and G-CSF [22]. The latter $CD34^+$ cells appeared to be, on average, larger than those from normal BM and, in general, had functional features of more mature progenitors [22]. Furthermore, PBSC mobilized with chemotherapy and G-CSF had higher concentrations of various progenitor cells than normal BM, whereas in the current study, PBSC mobilized with G-CSF alone appeared to have the same or lower numbers of progenitor cells than normal BM. This observation confirms studies showing that mobilization with chemotherapy and G-CSF results in pheresis products richer in $CD34^+$ and clonable progenitor cells than mobilization with G-CSF alone [1, 30,31]. To *et al.* found that $CD34^+$ cells mobilized with G-CSF were more frequently $CD38^-$ than were $CD34^+$ cells mobilized with chemotherapy plus G-CSF [32]. This finding is compatible with the cells' elutriation characteristics, attesting to their smaller size observed in the current study (Fig. 2) [22, 23].

Mature T cells, defined as $CD3^+$ cells, are elutriated at low flow-rates, which has been the basis for the clinical use of elutriation procedures for T-cell depletion of bone marrow [15–19]. Previous studies demonstrated that early progenitor cells, such as HPP-CFC and LTC-IC, are elutriated at lower flow rates than more mature progenitor cells (e.g., CFU-GM) and the bulk of $CD34^+$ cells [20,21]. Consequently, T-cell depletion by elutriation may lead to an even more extensive loss of early progenitor cells than estimated by the loss of $CD34^+$ cells or CFU-GM, which are commonly used as clinical parameters [21]. The current study suggests that the same risk exists for MoPBSC. If the experimental method for this study were used to remove 96% of $CD3^+$ cells from MoPBSC products, then 63% of $CD34^+$ cells and CFC, but 85% of LTC-IC, would be lost (Fig. 3). Similarly, if 96% of $CD3^+$ cells were removed from BM products, 42% of $CD34^+$ cells, 49% of CFC, and 83% of LTC-IC would be removed. The loss of LTC-IC appears to be similar for T-cell depletion of MoPBSC and BM by this elutriation method, but the loss of more mature progenitor cells may be more pronounced for MoPBSC. Assuming BM and MoPBSC products each contain 5×10^6 $CD34^+$ cells/kg, and aiming at 96% T-cell depletion for both, the final products for infusion would contain quite different numbers of T cells. The final MoPBSC product would contain 1.8×10^6 $CD34^+$/kg, 2.1×10^8/kg nucleated cells, and 5.7×10^6/kg $CD3^+$ cells. The final BM graft would also contain 1.8×10^6 $CD34^+$/kg, but only 0.45×10^8/kg nucleated cells and 0.8×10^6/kg $CD3^+$ cells.

Other T-cell depletion strategies for MoPBSC, such as positive selection of $CD34^+$ cells or depletion of lymphocytes with the CAMPATH I monoclonal antibody, have led to similar losses of $CD34^+$ cells [33]. Despite the rapid expansion in the use of allogeneic PBSC, a significant risk for loss of early progenitor cells during elutriation procedures and other T-cell depletion remains. Relative risks and benefits must be weighed for each method in selecting a clinical strategy. It should be appreciated, however, that $CD34^+$ cell numbers, and even CFC, are inadequate surrogate

markers for the true hematopoietic stem cell, and that the contributions of various subsets of these surrogate markers on early and late engraftment still need to be ascertained in clinical allogeneic transplantation.

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